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ECDYSTEROID TITERS DURING THE MOLT CYCLE OF THE BLUE CRAB RESEMBLE THOSE OF OTHER CRUSTACEA

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Abstract

Callinectes sapidus is the only true crab (brachyuran) whose pattern of ecdysteroid titers has been described as departing from the pattern seen in other decapods. While ecdysteroids in other crabs reach a peak just prior to ecdysis, those of *C. sapidus* were claimed to reach their maxima after ecdysis. The data reported here challenge these findings. We have measured ecdysteroids in hemolymph, ovaries, and whole animal extracts of blue crabs using a radioimmunoassay. In hemolymph and whole animals, ecdysteroid levels rose during premolt to a maximum at stage D_3. Ecdysteroids declined rapidly from late premolt stage D_4 through postmolt stage A_2, increased slightly at postmolt stage B, and returned to low levels where they remained during intermolt stage C. Ecdysteroid levels in males and immature females were not significantly different but mature females, having reached a terminal anecdysis, had significantly lower ecdysteroid levels. Ovaries of mature females accumulated ecdysteroids during vitellogenesis while the concentration of ecdysteroids in hemolymph was low.

Introduction

Ecdysteroids in crustaceans, measured in whole animals or hemolymph, rise during proecdysis, reach peak levels shortly before ecdysis, and decline to basal levels before or soon after ecdysis (Spindler *et al.,* 1980; Skinner, in press). This pattern is consistent with the role of 20-hydroxyecdysone (20HE) in initiating premolt. When ecdysteroids were examined in female blue crabs *Callinectes sapidus,* 20HE, inokosterone, and makisterone A were identified and, surprisingly, the ecdysteroid peak, consisting principally of 20HE, occurred after ecdysis (Faux *et al.,* 1969). It was suggested that the hormone peak during postmolt was involved with hardening of the exoskeleton (Faux *et al.,* 1969). Because of the decline in hormone titers following ecdysis in the crayfish *Orconectes limosus,* Willig and Keller (1973) concluded that calcification of exoskeleton was independent of hormonal control.

Until the experiments described here, there has been no investigation of circulating ecdysteroid titers nor of ecdysteroids in individual tissues of *C. sapidus.* These are important data since many arthropods regulate ovarian maturation and embryonic development by sequestering ecdysteroids in the ovaries during the reproductive stage; regulation of the molt cycle is distinguished by changes in circulating ecdysteroids. Several insects accumulate ecdysteroids in the ovary (Garen *et*

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al., 1977; Lagueux *et al.*, 1977; Hoffman *et al.*, 1980) as do the crabs *Carcinus maenas* (Lachaise and Hoffman, 1977) and *Acanthonyx lunulatus* (Chaix and De Reggi, 1982). Although *Carcinus* continues to molt after its reproductive phase, *Acanthonyx* and other oxyrhynchans enter a terminal anecdysis (stage C_4T; Carlisle, 1957) at the puberty molt. Similarly, *Callinectes*, a brachyrhyncan, enters terminal anecdysis after reaching the puberty molt (Churchill, 1919). It was therefore important to determine ecdysteroid concentrations in both hemolymph and ovaries of crabs in this terminal anecdysis. To that end we examined the ecdysteroid titers in hemolymph, ovaries, and whole animals at different stages of the molt cycle using a radioimmunoassay (RIA; Soumoff *et al.*, 1981). We compared males and females to determine whether there were any hormonal differences between sexes and compared sexually immature females which still undergo ecdyses with sexually mature females that are in a terminal anecdysis.

Materials and Methods

Animals

Crabs were collected off the Virginia coast during June and July of the molting season. They ranged in size from 6.3 cm to 11.4 cm carapace width. Animals collected in various phases of the molt cycle were staged by the coloration on the distal segments of the swimming legs (Churchill, 1919) and by the extent of skeletal resorption at the epimeral suture (Warner, 1977; Passano, 1960). Initially, four stages were examined: intermolt (C_4), early premolt (D_1 or green crabs), late premolt (D_3 or peeler crabs) and postmolt (A_1–B_2 or soft crabs). A second series of experiments examined crabs divided into several substages from A_1 through D_4 (see Passano, 1960; Skinner, 1962; Warner, 1977 for descriptions of stages). Mature females, immature females, and males were distinguished by the characteristic shapes of the abdomen.

Treatment of biological material

Hemolymph was withdrawn by syringe puncture through the pericardial space, the arthrodial membrane at the base of a limb, or the mid-joint of a claw. Clotted hemolymph was disrupted and centrifuged to obtain serum. Aliquots were taken for radioimmunoassay (RIA) and the remaining serum was pooled by stage and sex. Ovaries and bursa copulatrix were excised from mature females, blotted dry, and weighed prior to exhaustive hemolymph removal or hemolymph, bursa, and ovary removal. Individual tissues or whole animals were homogenized in 75% MeOH and centrifuged. Pellets were reextracted in 75% methanol and supernatants were evaporated under reduced pressure and resuspended in a small volume of 75% methanol. Samples were examined by RIA.

Radioimmunoassay

Antiserum was that of Soumoff *et al.* (1981) produced from 20-hydroxyecdysone 2-hemisuccinate conjugated to thyroglobulin. [^{3}H]ecdysone (S.A. 50 Ci/mmol or 80 Ci/mmol) was the tracer ligand. 20HE (Simes, Italy) was used as a standard to estimate ecdysteroid levels. All titers are given as 20HE equivalents, although the antiserum has different reactivities toward closely related ecdysteroids (Soumoff *et al.*, 1981). The RIA protocol has been described elsewhere (Chang and O'Connor, 1979).

RESULTS

An initial survey revealed that serum ecdysteroids were at basal levels in intermolt crabs, began rising in early premolt crabs, and reached peak titers in late premolt crabs (Fig. 1A). By postmolt serum titers dropped, but not as low as intermolt levels. Males and females showed no statistically significant differences at any given stage. Variance was greater among males than females and was not related to size or limb loss. Blue crabs readily autotomize limbs as a result of handling; most of the animals lost from 1 to 4 limbs while two crabs lost six limbs. Regenerating limb buds from previously autotomized limbs were small on intermolt crabs but

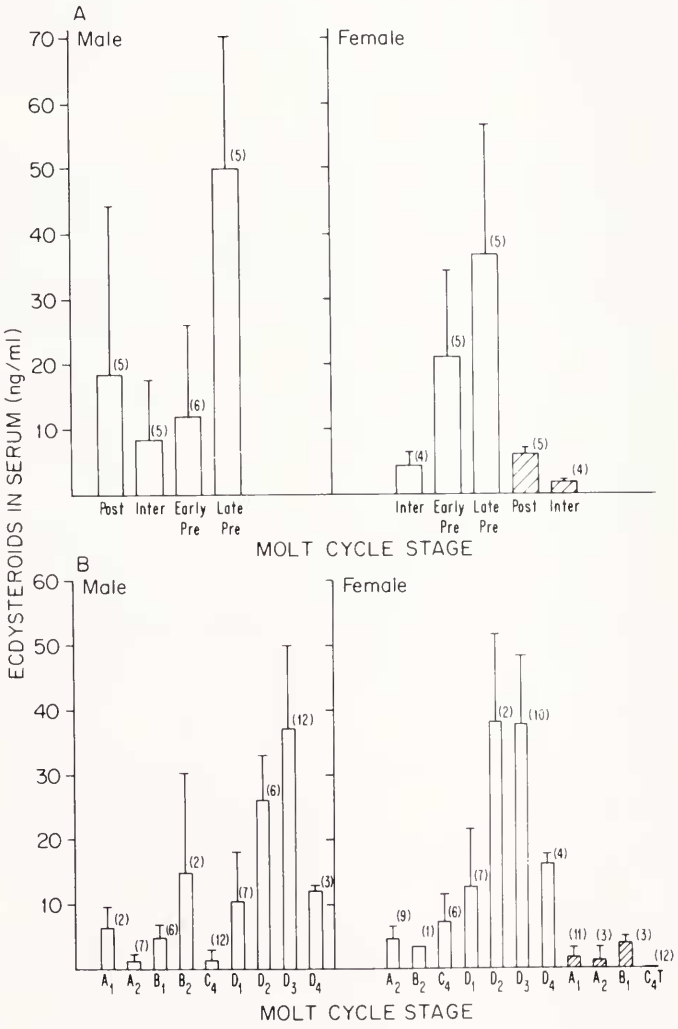

FIGURE 1. Serum ecdysteroid levels during the molt cycle in male and female blue crabs collected in (A) June, 1981 and (B) June, 1982. Values are the means ± standard deviations. Number of animals assayed are given in parentheses. Hatched bars represent mature females. Ecdysteroids were calculated as 20HE equivalents.

were large on premolt crabs. It has been shown that ecdysteroid titers are elevated in crabs in advanced stages of limb regeneration (Soumoff and Skinner, 1980). Multiple autotomy acts as a stimulus to molt (Skinner and Graham, 1970, 1972; Holland and Skinner, 1976; Mykles and Skinner, 1981) and limb regeneration is a sign that a crab is in the premolt stage (Emmel, 1906, 1907; Bliss, 1956).

Since the puberty molt is the final molt for females of this species, mature females are found only in the postmolt and subsequent C_4T stages. Although immature females should be available in all stages of the molt cycle, we were unable to obtain postmolt immature females during this initial survey. Mature C_4T females had lower serum ecdysteroids than immature intermolt females. The difference was significant ($P < .05$) and is probably related to changes in hormone production and metabolism causing the terminal anecdysis of mature females. In one case an immature female was assayed in late premolt, completed the molt to maturity overnight, and was reassayed in postmolt. The premolt ecdysteroid level, 43.4 ng/ml, decreased to 6.7 ng/ml overnight.

A second examination of serum ecdysteroid levels was undertaken during the next annual molting season (Fig. 1B) and the molt cycle stages were defined more precisely. The observed hormone levels confirmed the data obtained previously (Fig. 1A). Ecdysteroid concentrations rose during the initial stages of premolt, declined in stage D_4 and continued to decline through stage A_2. There was a slight rise in ecdysteroid concentration in stage B_1. The apparent rise in stage B_2 males was caused by one exceptionally high value that may have been an artifact. There were no significant differences between males and females throughout premolt. Mature females had significantly lower ecdysteroid levels than immature females at stages A_2 and C ($P < .05$) and males at stages A_1 and C ($P < .02$). Among thirteen mature C_4T females examined, twelve showed no detectable ecdysteroids and one had a level of 5 ng/ml. Intermolt juvenile females averaged 7.1 ng/ml and intermolt males averaged 1.3 ng/ml.

Some crabs that survived several premolt and postmolt stages in captivity were sampled in consecutive stages. Figure 2A shows that serum ecdysteroids rose in individual specimens as they proceeded from stage D_1 to stage D_3. Crabs that were collected at later premolt stages had rapidly declining serum ecdysteroids (Fig. 2B). These data illustrate that although there may be wide variations between crabs, a pattern is maintained within individuals of rising ecdysteroids through stage D_3 and declining ecdysteroids from stage D_4 through A_2.

In several species of insects (Luu *et al.*, 1976; Lagueux *et al.*, 1977; Ohnishi *et al.*, 1977; Bollenbacher *et al.*, 1978) and in the crab *C. maenas* (Lachaise and Hoffmann, 1977) reproductively active ovaries contain ecdysteroids which regulate vitellogenesis (Hagedorn *et al.*, 1975; Handler and Postlethwait, 1978) and embryonic development (Hoffmann *et al.*, 1980). We examined the ecdysteroid concentration in ovaries of mature female blue crabs to determine whether they stored significant amounts of ecdysteroids. As a control tissue we examined the bursa copulatrix, the storage sacs for sperm introduced during copulation.

The reproductive stages were determined according to criteria which distinguish changes in the gross appearance of the ovaries (Hard, 1942). Stage I describes crabs immediately following the puberty molt when ovaries are small. Stage II describes the period during which the ovary enlarges and becomes orange as vitellogenesis progresses. Stage III describes the mature ovary which is very large and bright orange.

The ecdysteroid content of ovaries of *C. sapidus* increased as vitellogenesis progressed (Table I) although ecdysteroid concentration per unit weight declined 2.5-

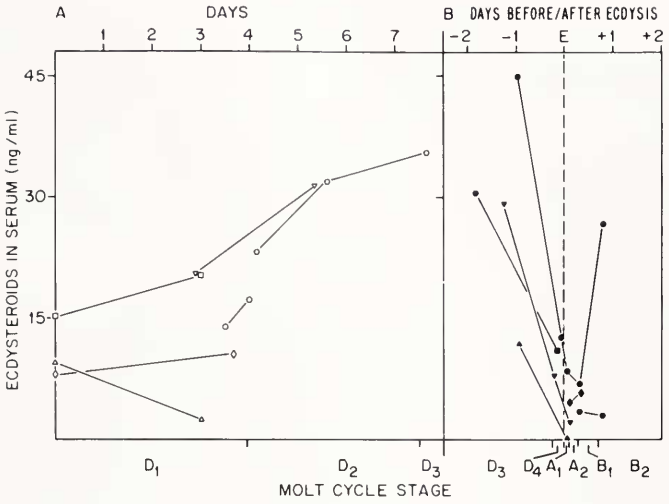

FIGURE 2. Serum ecdysteroid levels in individual crabs at consecutive stages of the molt cycle. Each symbol represents a single crab whose serum was examined at the intervals shown. At each interval, the stage of the cycle was determined by the condition of the exoskeleton and coloration of an appendage. (A) Crabs in stages D_1 through D_3. The upper axis shows the number of days between measurements. (B) Crabs in stages D_3 through B_2. All animals reached ecdysis. The upper axis shows the number of days between measurements in relation to the time of ecdysis.

fold during yolk deposition as the weight of the ovary increased almost thirty-fold. In contrast, ecdysteroids in the closely associated bursa copulatrix decreased from stage I to stage III. Ecdysteroid accumulation in the ovaries of C_4T females occurred at a time when ecdysteroids were low in both serum (Fig. 1) and whole animals (Table II). Although ovaries accumulated ecdysteroids during vitellogenesis, their content of ecdysteroids did not contribute significantly to the whole animal titer.

Total ecdysteroid content in both males and females rose to maximum levels during late premolt and declined precipitously by postmolt (Fig. 3). The pattern of ecdysteroid titers measured throughout the molt cycle is similar to the pattern for serum or carcass alone. These results are contrary to those of Faux *et al.* (1969) who observed maximal ecdysteroids during postmolt in whole animal extracts of females.

TABLE I

Ecdysteroid levels in female reproductive tissue

Tissue	Stage	*N*	Weight (mg/organ pr)	Ecdysteroid Conc. (ng/organ pr)	Ecdysteroid Conc. (ng/g)
Ovary	I	5	130 ± 20	0.35 ± 0.12	2.86 ± 1.19
	II	3	660 ± 80	1.39 ± 0.12	2.14 ± 0.44
	III	3	3240 ± 40	3.56 ± 1.09	1.10 ± 0.35
Bursa Copulatrix	I	5	710 ± 190	3.35 ± 1.38	4.70 ± 1.57
	II	3	1120 ± 620	1.58 ± 1.01	1.45 ± 0.36
	III	3	180 ± 60	0.54 ± 0.32	3.22 ± 1.27

TABLE II

Mature female whole animal ecdysteroids

Stage	*N*	Weight (g)	Ecdysteroid (ng/g)
A_1	4	94.08 ± 11.06	6.34 ± 2.25
C_4T	6	117.73 ± 19.99	2.48 ± 1.19

DISCUSSION

Contrary to previous results in which ecdysteroids reached a peak after ecdysis (Faux *et al.*, 1969) the results described here indicate that ecdysteroid concentrations in *Callinectes sapidus* are at basal levels during intermolt, increase an average of seven-fold by late premolt, and decline in postmolt. Whole animal ecdysteroid titers for both sexes average 10.4 ng/g fresh weight, 74.8 ng/g fr. wt. and 15.8 ng/g fr. wt. respectively at these stages. The antiserum we used has varying sensitivity toward different ecdysteroids. It is three-fold more sensitive to ecdysone than to 20HE while its sensitivity toward all other ecdysteroids tested is less than that to 20HE (Soumoff *et al.*, 1981). This will have some effect on measurements of complex mixtures of ecdysteroids. The concentrations we observed, however, are consistent with ecdysteroid levels in other crustaceans. Titers measured in the crab *Carcinus maenas* (Adelung, 1969) range from 5 ng/g at intermolt to 110 ng/g during premolt. In the amphipod *Orchestia gammarella*, the range is from 12 ng/g at intermolt to 63 ng/g at late premolt (Blanchet *et al.*, 1976). Ecdysteroids in the crayfish *Orconectes limosus* range from 0.3 ng/g during intermolt to 60 ng/g during premolt (Willig and Keller, 1973). In adult female lobsters (*Homarus americanus*) ecdysteroids are 6 ng/g at postmolt (Gagosian *et al.*, 1974). Quantitation of the values for *Orchestia* was by RIA, for *Carcinus* and *Orconectes* by bioassay, and for *Homarus* by high pressure liquid chromatography and gas chromatography. Although the method of quanti-

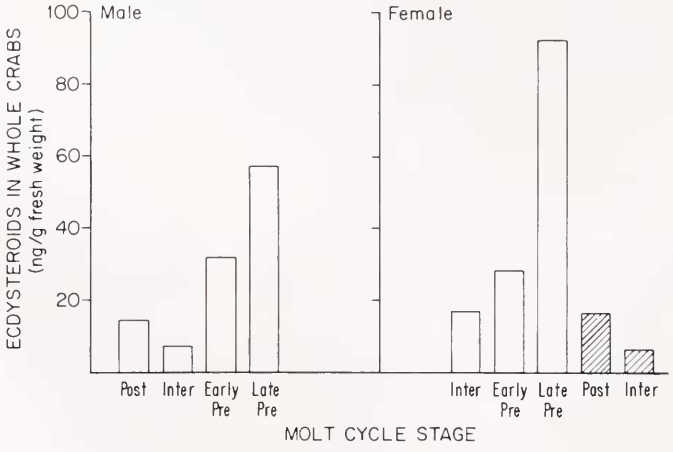

FIGURE 3. Whole animal ecdysteroid levels in male and female blue crabs at different stages of the molt cycle. Hatched bars represent mature females. Ecdysteroids were calculated as 20HE equivalents. Three or four animals from each stage were pooled and assayed. Hemolymph from both sexes and ovary and bursa from mature females at each stage were assayed separately from remaining carcass and the values were added to calculate the titers in whole animals.

tation determines, to some extent, the titer of hormone measured, these examples, utilizing several different techniques, are consistent with each other.

Ecdysteroids measured by Faux *et al.* (1969) for female blue crabs are inconsistent with the values reported here. In that analysis, the peak of ecdysteroids was observed after ecdysis (280 ng/g 20HE and 24 ng/g makisterone A) and was twelvefold greater than the concentration at late premolt (20 ng/g inokosterone and 4 ng/g 20HE). The method of quantitation of ecdysteroids was not specified and may account for the discrepancy. One other example of a major peak of hormone titer during postmolt was reported for *O. gammarella* (Blanchet *et al.*, 1976). The hormone titer reached a maximum in late premolt, declined by stage A, but showed some indication of a second peak during stage B; a large standard deviation at this stage made interpretation of the data difficult.

Measurements of circulating ecdysteroids are more variable between species than are whole animal titers. However, all species exhibit a trend of increasing ecdysteroid levels during premolt to a maximum prior to ecdysis, followed by a decline to basal intermolt levels. The range of ecdysteroids in *Callinectes* serum, 5 ng/ml at intermolt to 44 ng/ml in late premolt, is comparable to hemolymph titers of the crayfish *Orconectes sanborni* ranging from 4 ng/ml to 30 ng/ml (Stevenson *et al.*, 1979). Ecdysteroids in hemolymph of the crab *Pachygrapsus crassipes* vary from near zero just after ecdysis to 120 ng/ml in premolt (Chang and O'Connor, 1978). The crab *Gecarcinus lateralis* has a minimal titer of 10 ng/ml at intermolt and a maximum of 150 ng/ml at D_3 when induced to molt by multiple limb autotomy (Soumoff and Skinner, 1982). Serum levels are in that same range in the fiddler crab *Uca pugilator* (Hopkins, In press) during a natural molt cycle. Lachaise *et al.* (1976) reported circulating ecdysteroid titers ranging from 62–470 ng/ml for the crab *C. maenas,* while titers of 30–15,000 ng/ml hemolymph for this species have also been reported (Andrieux *et al.*, 1976). Juvenile lobsters, *Homarus americanus,* exhibited basal levels of ecdysteroids of less than 35 ng/ml and peak titers of 350 ng/ml (Chang and Bruce, 1980). These values were all quantitated by RIA.

Whole animal and serum ecdysteroid titers in mature *Callinectes* females during postmolt were significantly higher than those in mature females at the subsequent intermolt stage. Despite this, intermolt ovaries contained higher levels of ecdysteroids than postmolt ovaries; the former were vitellogenic while the latter were not. Similarly, the ecdysteroid concentration in ovaries increased at vitellogenesis while the ecdysteroids in hemolymph remained low in *C. maenas* (Lachaise and Hoffmann, 1977) as well as in the spider crab *Acanthonyx lunulatus* (Chaix and de Reggi, 1982).

Females of the oxyrhynchan species *Maja squinado* and *A. lunulatus* reach reproductive maturity at their last molt, when they enter terminal anecdysis. Their Y-organs become inactive and degenerate (Carlisle, 1957; Chaix *et al.*, 1976) and hemolymph ecdysteroids decline (Chaix and de Reggi, 1982). Similarly for male isopods (*Sphaeroma serratum*), the Y-organs degenerate following the puberty molt, a terminal anecdysis is reached, and ecdysteroids gradually disappear from the hemolymph (Charmantier, 1980). The very low hemolymph ecdysteroids in mature C_4T females of *C. sapidus* is consistent with these observations and, similarly, may result from degenerative changes in the Y-organs.

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